

## Involvement of Prostaglandin E Receptor Subtype EP<sub>4</sub> in Colon Carcinogenesis<sup>1</sup>

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### Abstract

Accumulating evidence indicates that overproduction of prostanoids attributable to overexpression of cyclooxygenase-2 (COX-2) plays an important role in colon carcinogenesis. We have shown recently that the prostaglandin (PG) E receptor, EP<sub>1</sub>, but not EP<sub>3</sub>, is involved in mouse colon carcinogenesis. In line with our previous study, here we examined the role of prostanoid receptors in colon carcinogenesis using six additional lines of knockout mice deficient in prostanoid receptors EP<sub>2</sub>, EP<sub>4</sub>, DP, FP, IP, or TP. The animals were treated with the colon carcinogen, azoxymethane (AOM), and examined for the development of aberrant crypt foci (ACFs), putative preneoplastic lesions in the colon. Formation of ACFs was decreased only in the EP<sub>4</sub>-knockout mice, to 56% of the wild-type level. To confirm these results, we also examined the inhibitory effects of an EP<sub>4</sub>-selective antagonist, ONO-AE2-227, in the diet on the formation of AOM-induced colon ACFs in C57BL/6Cr mice and on the development of intestinal polyps in Min mice. ONO-AE2-227 at a dose of 400 ppm reduced the formation of ACFs to 67% of the control level, and intestinal polyp numbers in Min mice receiving 300 ppm were decreased to 69% of the control level. Plating efficiency assays showed that addition of 1.0  $\mu$ M ONO-AE1-329, an EP<sub>4</sub>-selective agonist, resulted in a 1.8-fold increase in the colony number of the human colon cancer cell line, HCA-7, similar to the effect of PGE<sub>2</sub>. Moreover, EP<sub>4</sub> mRNA expression was clearly observed in normal colon mucosa and colon tumors in mice. Our previous and present results indicate that PGE<sub>2</sub> contributes to colon carcinogenesis through its actions mediated through EP<sub>1</sub> and EP<sub>4</sub> receptors; therefore, antagonists for these two receptors may be good candidates as chemopreventive agents against colon cancer.

### Introduction

Clear benefits have been reported with NSAIDs<sup>4</sup> as chemopreventive agents against colon carcinogenesis (1, 2). NSAIDs inhibit arachidonic acid metabolism via actions on COX, a rate-limiting enzyme in the synthesis of PGs, which affect cell proliferation, tumor growth, apoptosis, and immune responsiveness. The presence of two isoforms of COX has been demonstrated—a constitutive enzyme, COX-1, and an inducible enzyme, COX-2—and a number of observations have

suggested that increased activity of this latter plays a critical role in colon carcinogenesis (1–6). Recently, it was reported that genetic disruption of COX-1, as well as of COX-2, significantly reduces intestinal polyp formation in Min mice having a nonsense mutation in the *Apc* gene (7), so that COX-1 is also suggested to be involved in colon carcinogenesis to some extent.

When considering the possible mechanisms for the chemoprevention of colorectal cancer by NSAIDs, account must be taken of possible PG-independent mechanisms. Studies have shown that NSAIDs cause an increase in cellular arachidonic acid and stimulate the production of sphingomyelinase, resulting in hydrolysis of sphingomyelin to ceramide, which promotes apoptosis of tumor cells (8). Recently, the potential involvement of peroxisome proliferator-activated receptor  $\delta$  as a adenomatous polyposis coli-regulated target of NSAIDs in colon cancer was demonstrated (9). Moreover, NSAIDs can up-regulate the *prostate apoptosis response 4* gene, a proapoptotic gene, in human colon carcinoma HCA-7 cells (10).

On the other hand, the most striking chemopreventive effects of NSAIDs are thought to be attributable to inhibition of COX with a resultant decrease in PG production. However, it is not fully clear what the legitimate molecular target of PGs is. Prostanoids such as PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , PGI<sub>2</sub>, and thromboxane A<sub>2</sub> exert their biological actions through binding to eight specific membrane receptors; the four subtypes EP<sub>1</sub> to EP<sub>4</sub> for PGE<sub>2</sub>; DP for PGD<sub>2</sub>; FP for PGF<sub>2 $\alpha$</sub> ; IP for PGI<sub>2</sub>; and TP for thromboxane A<sub>2</sub> (11, 12). The recent establishment of mice lacking the genes encoding these receptors (13–18) has enhanced our understanding of the involvement of prostanoids and their receptors in the development of colon cancer. In previous studies (19, 20), we demonstrated that PGE<sub>2</sub> contributes to colon carcinogenesis through its binding to the PGE<sub>2</sub> receptor subtype EP<sub>1</sub>, using a genetic approach in EP<sub>1</sub>-knockout mice and a pharmacological assessment with the EP<sub>1</sub>-selective antagonists, ONO-8711 and ONO-8713. The same approach using EP<sub>3</sub>-knockout mice indicated that the deficiency of EP<sub>3</sub> receptor has no effect on colon carcinogenesis (19).

The present study was conducted to examine the development of ACFs in six additional lines of mice lacking EP<sub>2</sub>, EP<sub>4</sub>, DP, FP, IP, or TP. Our results indicate a requirement for the EP<sub>4</sub> receptor in ACF formation by AOM. To confirm these data, we also examined the inhibitory effects of an EP<sub>4</sub>-selective antagonist on the formation of colon ACFs induced by AOM in C57BL/6Cr mice and on the development of intestinal polyps in Min mice. Moreover, we determined EP<sub>4</sub> mRNA expression in colonic tissues of mice and examined cell proliferative effects of EP<sub>4</sub> receptor activation using an EP<sub>4</sub>-selective agonist. On the basis of the results obtained, the role of EP<sub>4</sub> receptor in colon carcinogenesis is discussed.

### Materials and Methods

**Animals.** Male C57BL/6Cr mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) at 5 weeks of age and female C57BL/6J-Min/+ mice (Min mice) from The Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age. The

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<sup>4</sup> The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PG, prostaglandin; ACF, aberrant crypt focus; AOM, azoxymethane; ONO-AE2-227, 2-[2-(2-(1-naphthyl)propanoylamino)phenyl]methylbenzoic acid; ONO-AE1-329, 16-(3-methoxymethyl)phenyl- $\omega$ -tetranor-3,7-dithia-PGE<sub>2</sub>; cAMP, cyclic AMP; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR.

mouse genes encoding each of the six prostanoid receptors, EP<sub>2</sub>, EP<sub>4</sub>, DP, FP, IP, and TP, were disrupted by a gene knockout method using homologous recombination, as reported previously (13, 15–18). The generated chimeric mice were mated with C57BL/6Cr mice to produce heterozygotes for the respective alleles. The heterozygotes were backcrossed with C57BL/6Cr mice to exclude possible effects of genetic background. The resulting heterozygous male mice were intercrossed, and the F<sub>2</sub> progeny of the wild-type and homozygous mutant mice were used at 9 (EP<sub>2</sub>, FP, IP, and TP) or 13 (DP) weeks of age. In the case of the EP<sub>4</sub>-knockout mice, chimeric mice were mated to C57BL/6Cr mice, and homozygous mutants were obtained by interbreeding of the resulting agouti offspring. Most EP<sub>4</sub>-deficient neonates with 129 × C57BL background become lethargic within 72 h after birth because of a patent ductus arteriosus, and <5% survive and grow normally (16). We used male EP<sub>4</sub>-knockout mice that survived for analysis of ACF formation starting at 7 weeks of age. Genotypes of the knockout mice were confirmed by PCR according to the method described previously (13, 15–18). The animals were housed in plastic cages at 24 ± 2°C and 55% relative humidity with a 12/12-h light/dark cycle. Water and basal diet (AIN-76A; Bio-Serv, Frenchtown, NJ) or experimental diets, with addition of an EP<sub>4</sub>-selective antagonist at the indicated concentrations with thorough mixing, prepared every week, were given *ad libitum*. Body weights and food intake were measured weekly. The experimental protocol was approved by the Institutional Ethics Review Committee for animal experimentation.

**AOM-induced ACF Formation in Prostanoid Receptor-Knockout Mice.** EP<sub>2</sub>, EP<sub>4</sub>, DP, FP, IP, and TP-knockout [EP<sub>2</sub><sup>−/−</sup> (male, *n* = 7), EP<sub>4</sub><sup>−/−</sup> (male, *n* = 10), DP<sup>−/−</sup> (female, *n* = 11), FP<sup>−/−</sup> (male, *n* = 9), IP<sup>−/−</sup> (male, *n* = 10), and TP<sup>−/−</sup> (male, *n* = 11)] mice and counterpart wild-type mice (*n* = 7–11/group) were treated with AOM (Sigma Chemical Co., St. Louis, MO) at a dose of 10 mg/kg body weight i.p. once a week for 3 weeks. All mice were sacrificed 5 weeks after the first dose of AOM. After laparotomy, the entire colons were resected and filled with 10% neutral buffered formalin and then opened longitudinally from the anus to the cecum. Each was fixed flat between sheets of filter paper in 10% neutral buffered formalin and then stained with 0.2% methylene blue in saline and scored under a light microscope for the number of ACFs/colon, number of ACs/colon, and mean number of ACs/focus according to the procedure of Bird (21).

**The Selective EP<sub>4</sub> Antagonist, ONO-AE2-227.** The selective EP<sub>4</sub> receptor antagonist, ONO-AE2-227, was chemically synthesized at Ono Pharmaceutical Co., Ltd. Receptor binding experiments with this compound were conducted using Chinese hamster ovary cell lines, stably expressing each type of mouse prostanoid receptor. The *Ki* values were found to be 2.7 nM for the mouse EP<sub>4</sub> receptor and 21 nM for mouse EP<sub>3</sub> receptor. The *Ki* values for the other receptors, mouse EP<sub>1</sub>, EP<sub>2</sub>, DP, FP, IP, and TP receptors were >1000 times higher than that for the mouse EP<sub>4</sub> receptor. Analysis of its agonistic and antagonistic actions showed the compound to act as a potent and competitive antagonist to the EP<sub>4</sub> receptor; it inhibited PGE<sub>2</sub> (100 nM)-induced increase in cytosolic cAMP concentration with a median inhibitory concentration of 10 nM. ONO-AE2-227 also acted as a relatively weak antagonist to the EP<sub>3</sub> receptor; it inhibited the PGE<sub>2</sub> (10 nM)-induced increase in cytosolic calcium concentration with an IC<sub>50</sub> of 160 nM. Details for the chemical synthesis and biological activities of ONO-AE2-227 will be reported elsewhere. By high performance liquid chromatography, ONO-AE2-227 was confirmed to be stable for at least 4 weeks at ambient temperature in the diet.

**Effects of ONO-AE2-227 on Formation of AOM-induced ACF in C57BL/6Cr Mice and Intestinal Polyps in Min Mice.** C57BL/6Cr male mice, 6 weeks of age, were given i.p. injections of AOM or the vehicle, as described in the experiments for the different lines of prostanoid receptor-knockout mice. The mice in the EP<sub>4</sub>-selective antagonist-treated groups were fed diets containing 100 or 400 ppm of ONO-AE2-227 starting the day before the first AOM dosing until the end of the experiment at week 5. Numbers of AOM-injected mice were eight for each group, and those for vehicle-injected mice were three for the 400 ppm of experimental diet groups and three for the control diet group. ACF in the colon of mice were assessed as described above.

Groups of 10 female Min mice were fed diet containing ONO-AE2-227 or basal diet from 6 weeks of age until the termination of the experiment 7 weeks thereafter. It is expected that C57BL/6Cr mice are generally much resistant to chemical treatment than Min mice. In addition, the experimental period with Min mice was longer than that of ACF induction in the experiment with C57BL/6Cr. Therefore, the dose of 300 ppm of ONO-AE2-227 was chosen for

the experiment. After sacrifice and laparotomy, the entire intestinal tract was resected, filled with 10% neutral buffered formalin, and divided into four sections: the colon and three sections of the small intestine, including the proximal (~4 cm from the pylorus ring of stomach), middle (the proximal half of the remainder), and distal parts. These sections were opened longitudinally and fixed flat between sheets of filter paper in 10% neutral buffered formalin, and the numbers and sizes of polyps were determined under a stereoscopic microscope.

**Effects of ONO-AE1-329 and PGE<sub>2</sub> on Colony Formation of HCA-7 Cells.** HCA-7 colony 29, a human colon adenocarcinoma cell line, was kindly provided from Dr. Susan Kirkland (Imperial College of Science, Technology and Medicine, London, United Kingdom). The cells were maintained in DMEM supplemented with 10% heat-inactivated FBS (Hyclone Laboratories, Inc., Logan, UT) and antibiotics (100 µg/ml of streptomycin and 100 units/ml of penicillin) at 37°C in 5% CO<sub>2</sub>. The numbers of HCA-7 cell colonies were counted as described previously (22) with slight modifications. In brief, HCA-7 cells were plated in 6-cm cell culture dishes at a density of 1000 cells/dish, with DMEM containing 10% FBS. The selective EP<sub>4</sub> receptor agonist, ONO-AE1-329 (23), or PGE<sub>2</sub> (Cayman Chemical Co., MI) was added daily to selected cells, and the medium was also replaced every day. Cells were incubated for 14 days, and then colonies were visualized by staining with 0.2% methylene blue and counted manually.

**Analysis of EP<sub>4</sub> Expression by RT-PCR in Colon Cancer Samples from AOM-treated Mice.** Male C57BL/6J mice (CLEA Japan, Inc., Tokyo, Japan) at 7 weeks of age were i.p. injected with 10 mg/kg body weight of AOM once a week for a total of six times to obtain many colon tumors, as described previously (3), and sacrificed at 50 weeks after the first injection. Their colons were removed, and one half of each of five colon tumors and five neighboring normal mucosa samples were immediately frozen, stored at -80°C, and used for RT-PCR analyses. The samples were sonicated, and total RNAs were isolated using ISOGEN (Nippon Gene Co., Tokyo, Japan). One-µg aliquots of total RNA were subjected to the reverse transcription reaction using an RNA LA PCR kit (Takara Shuzo Co., Shiga, Japan). Oligonucleotide primers specific for mouse EP<sub>4</sub> (5'-TTCCGCTCGTGGTGCAGTGTTC-3'; 5'-GAGGTGGTGTCTGCTTGGTCAG-3') were used for amplification of each mRNA. All PCR reactions were performed in a final volume of 50 µl for 30 cycles. The PCR products were analyzed by 2% agarose gel electrophoresis.

**Statistical Analysis.** The data for ACF and polyp formation are expressed as mean ± SE, and their statistical analysis was carried out with the Student's *t* test. Differences were considered statistically significant at *P* < 0.05.

## Results

**ACF Development in Prostanoid Receptor-Knockout Mice.** To determine which prostanoid receptors might be involved in colon carcinogenesis, we used a short-term *in vivo* model using ACF formation induced by AOM as the end point. The mean body weights of the AOM-treated EP<sub>4</sub>-knockout mice were comparable with those of AOM-treated wild-type mice. No abnormal clinical signs were observed during the course of the experiment, and no change was evident in organ weights including the liver, kidneys, and spleen between the two groups. ACFs were noted in all animals treated with AOM and were located mainly in the distal colon, with fewer numbers in the middle colon and rectum. In wild-type (EP<sub>4</sub><sup>+/+</sup>, *n* = 10) and knockout (EP<sub>4</sub><sup>−/−</sup>, *n* = 10) mice, the numbers of ACFs/colon were 14.6 ± 2.0 and 8.2 ± 1.4 (*P* < 0.05), and the mean numbers of ACs/focus were 1.5 ± 0.1 and 1.5 ± 0.1, respectively. Thus, the numbers of ACFs per colon in EP<sub>4</sub>-knockout mice were significantly reduced to 56% of the wild-type value. Mice treated with saline showed no evidence of ACF formation in either knockout or wild-type mice.

Under the same conditions, the effects of deficiency of EP<sub>2</sub>, DP, FP, IP, or TP receptors on formation of ACFs were examined. As with the EP<sub>4</sub>-knockout mice, no abnormal changes in body or organ weights were observed in knockout mice compared with wild-type mice, except for a slight increase in spleen weights of IP-knockout mice. There were no significant differences in the numbers of ACFs/

colon in EP<sub>2</sub>, DP, FP, IP, and TP-knockout mice from those of their wild-type counterparts. Moreover, the mean numbers of ACs/focus in these receptor-knockout mice groups did not differ from those in the wild-type mice. Fig. 1 summarizes the data for the effects of six prostanoid receptor deficiencies on AOM-induced ACF in mice. For reference, the results for EP<sub>1</sub>- and EP<sub>3</sub>-knockout mice, reported previously (19), are also included in Fig. 1.

**Suppression of AOM-induced ACF Formation by the EP<sub>4</sub>-selective Antagonist in C57BL/6Cr Mice.** To confirm a role for the EP<sub>4</sub> receptor in colon carcinogenesis, we investigated the effects of ONO-AE2-227, a selective EP<sub>4</sub> antagonist, on the formation of ACF induced by AOM in C57BL/6Cr mice. Administration of diet containing 100 or 400 ppm of ONO-AE2-227 did not affect the body and organ weights in the AOM-injected groups. ACFs were observed in all animals ( $n = 8$  for each group) treated with AOM. Administration of 400 ppm of ONO-AE2-227 to AOM-treated mice throughout the experiment for 5 weeks significantly decreased the numbers of ACFs/colon ( $8.3 \pm 1.1$ ,  $P < 0.05$ ) to 67% of that ( $12.4 \pm 2.0$ ) for the AOM-alone group. The mean numbers of ACs/focus in the two groups were both  $1.5 \pm 0.1$ . The numbers of ACFs/colon and the mean numbers of ACs/focus in 100 ppm of the ONO-AE2-227 group were  $12.4 \pm 1.6$  and  $1.5 \pm 0.1$ , respectively. Thus, administration of 100 ppm of ONO-AE2-227 did not affect ACF formation. No ACFs were observed in vehicle-injected mice, with or without 400 ppm ONO-AE2-227.

**Suppression of Intestinal Polyp Formation by the EP<sub>4</sub>-selective Antagonist in Min Mice.** Administration of ONO-AE2-227 at a dose of 300 ppm in the diet for 7 weeks did not affect the body weights, feeding, or behavior of Min mice. Data for number and distribution of intestinal polyps in the basal diet and ONO-AE2-227 groups are shown in Table 1. Most polyps were located in the small intestine with only a few in the colon. Administration of ONO-AE2-227 significantly reduced the total number of polyps to 69% of that in the basal diet group. The number of polyps detected in the distal portion of the small intestines was significantly lower (65% of the basal diet group value), and that in the middle portion was also lower (74% of the basal diet group value), although this was not statistically significant. Fig. 2 shows the size distributions of intestinal polyps in the basal diet and ONO-AE2-227-treated groups. Treatment with the EP<sub>4</sub>-selective antagonist significantly reduced the number of polyps

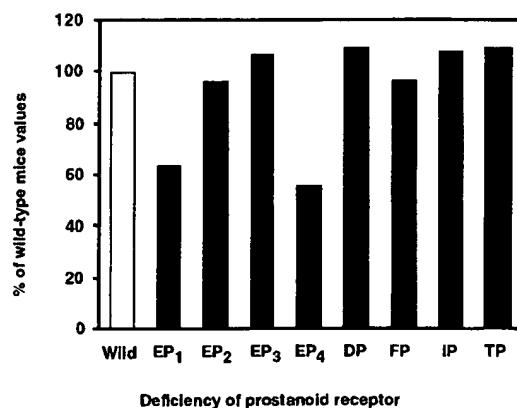


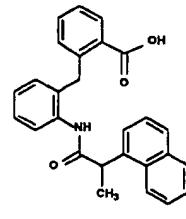
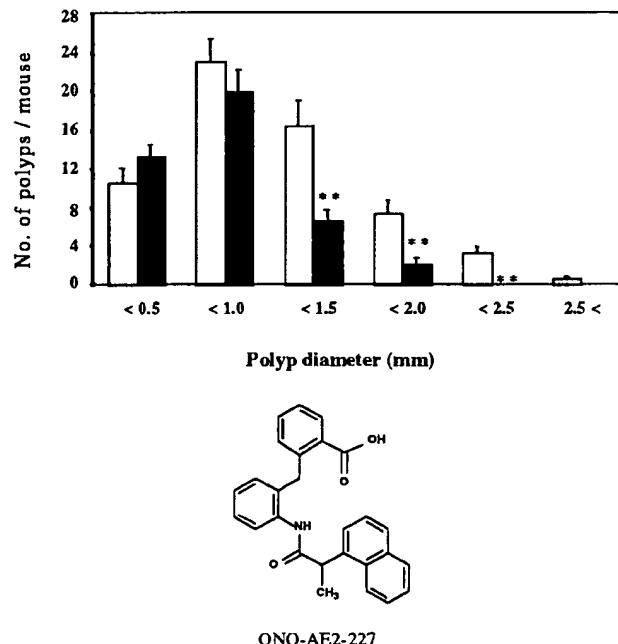
Fig. 1. Numbers of ACFs developing in the colons of prostanoid receptor-knockout mice and their wild-type counterparts after treatment with AOM. EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub>, EP<sub>4</sub>, DP, FP, IP, and TP-knockout mice and their respective wild-type mice were treated with AOM, and the numbers of ACFs were scored at 5 weeks after the first dose of AOM. The values (numbers of ACFs/colon) for each knockout mouse group are expressed as percentages of those for their wild-type counterparts. The data for EP<sub>1</sub>- and EP<sub>3</sub>-knockout mice are from our previously published work (19). □, wild-type mice; ■, knockout mice. \*,  $P < 0.05$ , compared with the wild-type mice.

Table 1 Suppression of intestinal polyp development by ONO-AE2-227 in Min mice

Mice were fed basal diet or diet containing 300 ppm of ONO-AE2-227 for 7 weeks. Numbers of Min mice fed the experimental and control diets were ten each. Data are mean  $\pm$  SE. Numbers in parentheses indicate percentage compared with the basal diet group.

Polyp location	No. of polyps/mouse	
	Basal diet	ONO-AE2-227
Proximal small intestine	$4.0 \pm 0.4$	$3.3 \pm 1.1$ (83)
Middle small intestine	$19.3 \pm 2.0$	$14.2 \pm 2.2$ (74)
Distal small intestine	$37.6 \pm 5.3$	$24.5 \pm 2.0^a$ (65)
Colon	$0.5 \pm 0.2$	$0.2 \pm 0.1$ (40)
Total	$61.4 \pm 7.3$	$42.2 \pm 4.3^a$ (69)

<sup>a</sup>  $P < 0.05$  versus the basal diet group.



ONO-AE2-227

Fig. 2. Size distribution of intestinal polyps in Min mice. □, basal diet; ■, 300 ppm ONO-AE2-227. (The structure is shown in lower part of the figure). Polyps were classified in terms of their diameters in millimeters. The number of polyps/mouse in each size class is expressed as the means; bars, SE. \*\*,  $P < 0.01$ , compared with the basal diet group.

measuring  $\geq 1.0$  mm in diameter but not those measuring  $< 1.0$  mm in diameter.

**Effects of the EP<sub>4</sub>-Selective Agonist and PGE<sub>2</sub> Treatment on Colony Formation of HCA-7 Cells.** To evaluate the physiological functions of the EP<sub>4</sub> receptor, we examined the effects of the EP<sub>4</sub> receptor-selective agonist, ONO-AE1-329, and PGE<sub>2</sub> treatment on colony formation in monolayer cultures. We used a human colon epithelial cell line, HCA-7 cells, in which expression of the EP<sub>4</sub> receptor could be detected by RT-PCR analysis (data not shown). For this experiment, 1000 cells were seeded in six-cm dishes, and ONO-AE1-329 was added daily at concentrations of 0.1, 1.0, and 10  $\mu$ M in fresh medium for 14 days. We observed a 1.8-fold increase in HCA-7 colony number in the presence of 1.0  $\mu$ M ONO-AE1-329 and a 1.5-fold increase in the presence of 1.0  $\mu$ M PGE<sub>2</sub> for 14 days (Fig. 3). The highest dose of ONO-AE1-329 (10  $\mu$ M) decreased the HCA-7 colony number.

**EP<sub>4</sub> mRNA Expression in Normal Colon and Colon Cancer Tissues in Mice.** The expression of EP<sub>4</sub> mRNA in normal colon mucosal and tumor tissues from five mice was examined. Representative data are shown in Fig. 4. EP<sub>4</sub> mRNA was detected in all colon tumor and normal mucosa samples by RT-PCR. All of the tumors

sampled were histopathologically diagnosed as well-differentiated adenocarcinomas.

## Discussion

In the present study, examination of the effects of EP<sub>2</sub>, EP<sub>4</sub>, DP, FP, IP, and TP receptor knockout on AOM-induced ACF formation in mice provided evidence for the involvement of the PGE<sub>2</sub> receptor subtype EP<sub>4</sub> but not EP<sub>2</sub>, DP, FP, IP, or TP in colon carcinogenesis. In addition, administration of an EP<sub>4</sub>-selective antagonist, ONO-AE2-227, to AOM-treated C57BL/6Cr mice and Min mice decreased ACFs and intestinal polyp formation, respectively. Interestingly, in the latter case the number of polyps  $\geq 1.0$  mm in diameter, but not those  $< 1.0$  mm in diameter, were reduced, suggesting reduction in tumor growth. An EP<sub>4</sub>-selective agonist, ONO-AE1-329, was further found to increase colony formation by HCA-7 cells, similar to PGE<sub>2</sub>. Moreover, we could demonstrate the expression of EP<sub>4</sub> receptors in colon tumors and normal mucosa, in line with earlier results of *in situ* hybridization for EP<sub>4</sub> mRNA (24). In the previous study, EP<sub>1</sub> receptor was shown to be involved in colon carcinogenesis (19). In addition, our preliminary study indicated that EP<sub>1</sub> receptor expression was detected in AOM-induced colon tumors in mice by RT-PCR analysis (data not shown). Thus, combined together, our present and previous results suggest that PGE<sub>2</sub> mediates carcinogenic changes by acting at EP<sub>1</sub> and EP<sub>4</sub> receptors in the colon. Consistent with these data, increased PGE<sub>2</sub> levels in colon tumor tissues compared with the surrounding normal mucosa were suggested to play an important role in colon carcinogenesis (25).

PGE<sub>2</sub> was earlier suggested to stimulate an increase in cell proliferation and motility of the colon cancer cell line LS-174 by activating the phosphatidylinositol 3-kinase/Akt pathway via EP<sub>4</sub> receptor activation (26). It is also known that PGE<sub>2</sub> activates adenylate cyclase via a cholera toxin-sensitive, stimulatory G protein through binding to the EP<sub>4</sub> receptor. In the adenylate cyclase pathway, increased cAMP levels result in an activation of cAMP-dependent protein kinase (PKA) and a transcriptional factor that binds to cAMP-responsive elements to transactivate the transcription of specific primary response genes that initiate cell proliferation (27). These biological changes could contribute to colon carcinogenesis through EP<sub>4</sub> receptor involvement. The EP<sub>1</sub> receptor is a transmembrane G protein-coupled receptor, similar to other PGE<sub>2</sub> receptors, but its signal transduction mechanism is not known in detail. EP<sub>1</sub> signals are transmitted by increased intracellular Ca<sup>2+</sup> concentrations and activate protein kinase C (11, 12). Additional studies are needed to

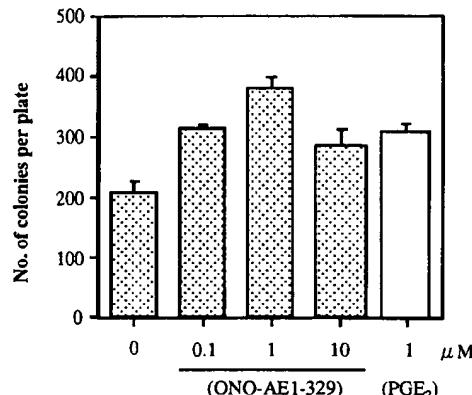


Fig. 3. HCA-7 colony number in response to treatment with ONO-AE1-329 or PGE<sub>2</sub>. HCA-7 cells were plated in six-cm cell culture dishes at a density of 1000 cells/dish, with DMEM containing 10% FBS. Cells were incubated for 14 days, and then colonies were visualized by staining with 0.2% methylene blue and counted manually. Data are means ( $n = 3$ ); bars, SE. Similar results were obtained in three separate experiments.

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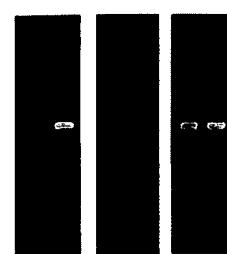


Fig. 4. EP<sub>4</sub> receptor expression in normal colon and colon cancer tissues of mice. Aliquots of total RNA (1  $\mu$ g) from colon cancer and neighboring normal colon tissues were subjected to RT-PCR. The reaction mixture for mouse EP<sub>4</sub> was first heated at 94°C for 2 min, followed by amplification for 30 cycles at 95°C for 30 s, 60°C for 45 s, and 72°C for 4 min. These temperatures and time intervals were followed by a final incubation at 72°C for 5 min. The PCR products were separated on 2% agarose gels, and portions corresponding to the expected DNA size (488 bp) of the PCR products are shown in the middle of the figure with an arrow. N, normal mucosa; T, tumor.

investigate events downstream of the EP<sub>1</sub> receptor signaling pathway and any link between EP<sub>1</sub> and EP<sub>4</sub> receptors. Recently, it was reported that homozygous deletion of the gene encoding EP<sub>2</sub> receptor resulted in decrease of intestinal polyp formation in the *Apc* knockout mice (28). These data are not consistent with the results obtained in the present study. Therefore, involvement of EP<sub>2</sub> receptor in AOM-induced colon carcinogenesis in rodents and intestinal polyp formation in Min mice needs to be examined using EP<sub>2</sub> receptor antagonists.

Selective inhibitors of COX-2 are good candidates as chemopreventive agents, with clinically important mechanism-based safety characteristics that significantly distinguish them from traditional NSAIDs, which suffer from gastrointestinal side effects that limit long-term application. It might be expected that these adverse effects are further diminished by inhibiting the downstream of COX pathway. On the basis of the present results, selective EP<sub>1</sub> and/or EP<sub>4</sub> receptor antagonists may be particularly beneficial as chemopreventive agents for colon cancer with low toxicity.

In conclusion, the data obtained in our present and previous studies suggest that PGE<sub>2</sub> mediates colonic carcinogenic changes by acting at EP<sub>1</sub> and EP<sub>4</sub> receptors in the colon. For confirmation, long-term colon carcinogenesis experiments with EP<sub>1</sub> and EP<sub>4</sub> antagonists are currently being conducted in our laboratory.

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